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Short-chain fatty acids can reduce binge-like eating behaviour in mice

Master's thesis

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Abstract

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Lyhytketjuiset rasvahapot voivat vähentää ahmintatyyppistä syömiskäyttäytymistä hiirillä.

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Abstract:

Binge eating disorder (BED) is a common eating disorder that includes eating a large amount of food in a short period of time and is often associated with obesity. Patients can suffer from stress, anxiety, and metabolic syndrome caused by the weight gain, but no effective medication for both psychological and physiological issues have been discovered. This thesis studies the potential of short-chain fatty acids (SCFA) in reducing binge-like eating behaviour in a mouse model that does not include food restriction. The model includes a 24 h bingeing period with high-fat food once a week. SCFA butyrate, propionate, and acetate were administered to mice via 1 g/kg i.p. injections or 200 mM drinking water for three days, and their effects on energy intake during the bingeing period were measured. The results show that SCFA can significantly reduce binge-like eating behaviour in mice in the short term, but long-term effects vary. I.p. butyrate, propionate, and acetate decreased energy intake by 68%, 57%, and 62% during the first hour, respectively. SCFA via drinking water did not decrease energy intake a lot, and the results were inconsistent between animals. These results suggest a potential for SCFA to attenuate bingeing episodes when administered acutely, but the mechanisms remain to be discovered.

Ahmintahäiriö (binge eating disorder, BED) on yleinen syömishäiriö, jonka tunnusmerkkinä on suurten ruokamäärien syöminen lyhyellä aikavälillä. Ahmintapotilaat voivat kärsiä stressistä, ahdistuksesta ja painonnousun aiheuttamasta metabolisesta oireyhtymästä, mutta psyykkisiin ja fysiologisiin haasteisiin ei ole löytynyt tehokasta lääkitystä. Tässä tutkielmassa tutkittiin lyhytketjuisten rasvahappojen (short-chain fatty acids, SCFA) potentiaalia vähentää ahmintatyyppistä syömiskäyttäytymistä hiirimallilla, johon ei liity ruoansaannin rajoittamista. Hiirimalliin kuuluu kerran viikossa tapahtuva 24 tunnin mittainen ahmintajakso, jonka aikana hiirille tarjotaan rasvaista ruokaa. Hiirille annettiin lyhytketjuisia rasvahappoja eli butyraattia, propionaattia tai asetaattia 1 g/kg intraperitoneaali-injektiona tai 200 mM juomaveden mukana kolmen päivän ajan, ja niiden vaikutus ahmintajakson aikaiseen energiansaantiin mitattiin. Saadut tulokset osoittavat, että lyhytketjuiset rasvahapot voivat merkittävästi vähentää ahmintatyyppistä syömiskäyttäytymistä hiirillä lyhyellä aikavälillä, mutta pitkän aikavälin vaikutukset vaihtelevat. Intraperitoneaalisesti annosteltu butyraatti, propionaatti ja asetaatti vähensivät ruoansaantia samassa järjestyksessä 68 %, 57 % ja 62 % ensimmäisen tunnin aikana. Juomaveden kautta annostellut rasvahapot eivät vähentäneet energiansaantia merkittävästi, ja tulokset olivat epäjohdonmukaisia eri hiirten välillä. Tulokset kuitenkin osoittavat, että lyhytketjuiset rasvahapot voivat mahdollisesti lieventää ahmintakohtauksia akuutisti annosteltuina, mutta mekanismien selvittäminen vaatii enemmän tutkimusta.

Contents

List of abbreviations

BED: binge eating disorder

SCFA: short-chain fatty acids

FFAR2: free fatty acid receptor 2

FFAR3: free fatty acid receptor 3

GPR: G protein-coupled receptor

HAT: histone acetyltransferase

HDAC: histone deacetylase

IL: interleukin

FFA: free fatty acids

ARC: arcuate nucleus

NTS: nucleus tractus solitarii

PYY: peptide YY

GLP-1: glucagon-like peptide 1

CCK: cholecystokinin

NGN: nodose ganglion neuron

DSM-5: Diagnostic and Statistical Manual of Mental Disorders 5

HFD: high-fat diet

i.p.: intraperitoneal

i.v.: intravenous

DW: drinking water

1. Introduction

1.1. What are short-chain fatty acids?

The gut microbiome provides the host with essential nutrients, including short-chain fatty acids (SCFA) that are produced by fermentation of dietary fibre. SCFA, especially butyrate and acetate, can also be received from the diet. SCFA are fatty acids with under 6 carbon atoms, and the most prevalent SCFA produced in the gut are butyrate, propionate and acetate with 4, 3 and 2 carbon atoms, respectively (Cummings *et al.*, 1987). They are all important for gut health and can function as signalling molecules and energy substrates. SCFA are present in many routes of the gut-brain-axis communication, but they also play many roles in metabolism on a systemic level (Canfora *et al.*, 2015).

SCFA are polar and therefore water-soluble metabolites that can be dissolved in blood without carriers, unlike the fat-soluble long-chain fatty acids. They are readily absorbed transcellularly from the gut lumen and enter the portal blood (Cummings *et al.*, 1987). Butyrate, propionate, and acetate have their unique effects, but they also share many functions. Of the three fatty acids, butyrate is mainly taken up by colonocytes, propionate by the liver and acetate by peripheral tissues where they can have different physiological effects and serve as energy substrates (Cummings *et al.*, 1987; Bergman, 1990; Koh *et al.*, 2016). They are all used effectively for maintenance, growth, and lipogenesis, even though they can be utilized in different ways. It has been estimated that butyric acid, propionic acid and acetic acid contain 5.92 kcal/g, 4.93 kcal/g and 3.48 kcal/g of energy, respectively (Livesey & Elia, 1988).

1.1.1. The receptors of SCFA

Many types of cells express G protein-coupled receptors that SCFA activate. FFAR (free fatty acid receptor) 2 and FFAR3 (also known as GPR43 and GPR41, respectively) are the most well-known SCFA receptors. Propionate is an effective activator of both receptors, while acetate is more selective on FFAR2 and butyrate on FFAR3 (Le Poul *et al.*, 2003), even though interspecies variability exists (Koh *et al.*, 2016). These receptors are expressed in many different tissues together or individually, including the intestine, adipose tissue, the peripheral nervous system, and the immune cells (Brown *et al.*, 2003; Kimura *et al.*, 2011; Nøhr *et al.*, 2013; Koh *et al.*, 2016). Another type of receptor, GPR109A, responds to butyrate and has been found to promote anti-inflammatory properties of macrophages and dendritic cells in the colon (Singh *et al.*, 2014). SCFA can also affect

cells via varied gene expression: they can act as histone acetyl transferase (HAT) activators or histone deacetylase (HDAC) inhibitors, depending on the cell type (Donohoe *et al.*, 2012; Koh *et al.*, 2016).

1.1.2. The effects of SCFA in the body

SCFA can improve gut health by providing energy to colonocytes, which promotes proliferation and enhances tight junctions between cells and therefore decreases permeability (Canfora *et al.*, 2015). Gut wall integrity is essential in preventing endotoxemia, in which bacterial lipopolysaccharides enter blood circulation causing low-grade inflammation. Endotoxemia could predispose to e.g. insulin resistance (Cani *et al.*, 2007). Different SCFA can also decrease or increase gut motility by e.g. regulating serotonin production, and activate secretory activity of enteroendocrine cells that hormonally signal satiety (Koh *et al.*, 2016; Stilling *et al.*, 2016). Both FFAR2 and FFAR3-dependent mechanisms and HDAC inhibition have their roles in these gut-related effects (Macia *et al.*, 2015; Koh *et al.*, 2016).

Lamina propria of the gut wall is filled with immune cells that are affected by SCFA via FFAR2 and GPR109A-dependent mechanisms. Treating mice with SCFA increases the number and activity of immune-suppressive T_{reg} cells, which are central regulators of antimicrobial immunity and tissue inflammation (Smith *et al.*, 2013). SCFA also enhance IL-18 production from proIL-18 in colonocytes, and this promotes tolerance to commensal bacteria and metabolites (Singh *et al.*, 2014; Macia *et al.*, 2015). Preventing dysbiosis of the gut microbiome with FFAR2 and GPR109A-dependent IL-18 production is important for the prevention of inflammation and epithelial disruption (Macia *et al.*, 2015; Canfora *et al.*, 2015). Activation of GPR109A by butyrate also increases the expression of anti-inflammatory IL-10 in dendritic cells and macrophages, which enhances T_{reg} cell differentiation (Singh *et al.*, 2014). In conclusion, SCFA activate mechanisms that help regulate the colonic immune response and reduce inflammation, while also maintaining a healthy gut epithelium and microbiome. It could also be said that SCFA are signalling agents produced by commensal bacteria that help maintain a beneficial milieu for them by affecting host physiology.

At least propionate and acetate can affect adipose tissue function via FFAR2-dependent ways. FFAR2 activation has been found to induce anti-lipolytic activity in adipocytes, enhancing lipid storage as triacylglycerols (Ge *et al.*, 2008). The reduced lipolysis can prevent lipid overflow of free fatty acids (FFA) into the blood, which was also seen in the same study with mice (Ge *et al.*, 2008).

Adipocyte differentiation can also be enhanced by SCFA (Li *et al.*, 2014). That promotes the healthy growth of adipocytes instead of hypertrophic growth (Longo *et al.*, 2019). Taken together, increased entry of fatty acids into adipocytes, decreased lipolysis, decreased plasma FFA and enhanced adipocyte differentiation all improve the buffering capacity of adipose tissue and decrease possible low-grade inflammation and insulin resistance.

1.1.3. Special properties of butyrate

Butyrate (Figure 1) is the most well-researched SCFA of the three. It can be derived from the diet from some dairy products like butter and some cheeses, but colonic fermentation is a more steady source of butyrate over time (Stilling *et al.*, 2016). Butyrate is the primary energy substrate in colonocytes, and only a small amount of butyrate ends up in the portal vein. Most of the remaining butyrate is cleared by the liver, making the circulating concentrations small (Cummings *et al.*, 1987). In the liver, butyrate can be metabolized via beta-oxidation or converted into ketone bodies.

Butyrate is important for gut epithelial health. It promotes the integrity of the gut wall by enhancing colonocyte proliferation and upregulating tight junction proteins leading to decreased permeability (Stilling *et al.*, 2016). Butyrate also suppresses tumor growth (Donohoe *et al.*, 2012). Since cancer cells use glucose as their energy source (the Warburg effect), butyrate accumulates more in cancerous cells and suppresses their proliferation by enhanced HDAC inhibition (Donohoe *et al.*, 2012). As stated earlier, butyrate is also the activator of the receptor GPR109A, which protects from colonic inflammation and is also thought to have a tumor-suppressive role (Singh *et al.*, 2014). The role of butyrate in HDAC inhibition, tumor suppression, and decreasing gut wall permeability is more well-known than that of propionate and acetate. It can be therefore stated that via butyrate, the commensal microbes in the gut provide the host with protection against colonic and systemic inflammation and colon cancer (Smith *et al.*, 2013).

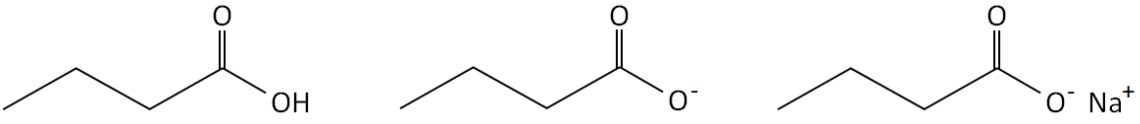


Figure 1. Butyric acid, butyrate, and sodium butyrate.

1.1.4. Special properties of propionate

Propionate (Figure 2) is not only produced by the gut microbiome, but it is also a product of beta-oxidation of fatty acids with an odd number of carbon atoms. After some modifications, propionate

can be incorporated into the TCA cycle and used for energy or gluconeogenesis. Colonic concentrations of propionate are similar to butyrate, but a majority of gut-derived propionate does not stay in colonocytes but ends up in the liver where it is metabolized for energy (Cummings *et al.*, 1987).

Propionate can decrease fatty acid synthesis and lipogenesis in hepatocytes and decrease cholesterol levels in rodents fed either propionate or substrates that increase microbial propionate production (Hosseini *et al.*, 2011). Propionate also has the potential to increase lipoprotein lipase (LPL) activity, which increases extracellular lipolysis and extracts fatty acids taking them into adipocytes (Canfora *et al.*, 2015). This enhances the storage of lipids. Propionate can also increase leptin production in adipocytes via FFAR3-mediated signalling (Xiong *et al.*, 2004). Leptin is a signal of a positive energy balance and can decrease food intake, which will be discussed later.

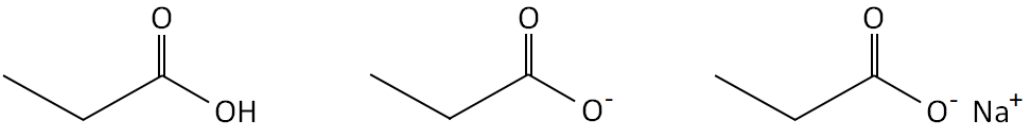


Figure 2. Propionic acid, propionate, and sodium propionate.

1.1.5. Special properties of acetate

Almost 60 % of the colonic SCFA content is acetate making it the most prevalent SCFA in the body. Compared to butyrate and propionate, more acetate ends up in the periphery, and it comprises about 90 % of the circulatory SCFA (Cummings *et al.*, 1987). Most of the acetate is still metabolized in the liver, but also other organs including heart, kidneys, adipose tissue and muscle take up acetate (den Besten *et al.*, 2013). Acetate (Figure 3) can be obtained from food as an additive, but highest amounts of acetate are found in vinegars that are 4-8 % acetic acid (González Hernández *et al.*, 2019). Acetate is also a breakdown product of ethanol. In the body, acetate is converted to acetyl-CoA, which can be used for energy or in the synthesis of some lipids or ketone bodies (den Besten *et al.*, 2013). Acetate has also been found to cross the blood-brain barrier (BBB) and affect food intake via a central homeostatic mechanism in mice (Frost *et al.*, 2014). Acetate directly effects the hypothalamic control of appetite by changing AMPK and ACC (acetyl-CoA carboxylase) activities, which causes changes in the expressions of downstream neuropeptides that are responsible for appetite regulation (Frost *et al.*, 2014).

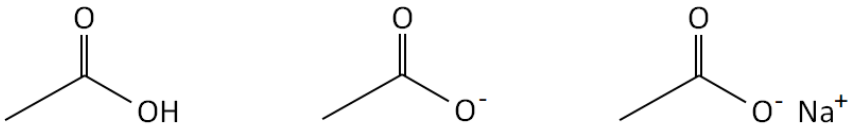


Figure 3. Acetic acid, acetate, and sodium acetate.

1.2. SCFA and satiety

The body aims at energy balance by regulating food intake via anorexigenic (satiety-inducing) and orexigenic (hunger-inducing) signalling. Both endocrine and neuronal signalling can regulate satiety and respond to energy-providing macronutrients (protein, fat, and carbohydrates) or mass of the food (increased by e.g. fibre). Short-chain fatty acids can increase satiety at least in murine animals by affecting both hormonal and neuronal satiety signalling.

1.2.1. Basic mechanisms of appetite and satiety regulation

The sense of satiety and appetite are complex phenomena that involve many brain areas and are not entirely understood. It is known that the hypothalamus is important for monitoring the bodily signals of energy balance. It senses the endocrine signals derived from e.g. the gastrointestinal tract and adipose tissue and delivers the message to other brain areas (Heisler & Lam, 2017). The arcuate nucleus (ARC) of the hypothalamus includes anorexigenic (i.e. satiety-inducing) and orexigenic (i.e. hunger-inducing) neuropopulations that produce their characteristic neuropeptides in response to endocrine signals. The signalling activity of these neuropopulations also affect each other, leading to the strengthening of either anorexigenic or orexigenic signalling (Heisler & Lam, 2017).

Endocrine signalling is essential in satiety regulation. Hormones that signal satiety and hunger are derived mainly from the gut and adipose tissue, but also e.g. insulin increases satiety. The gastrointestinal tract has its autonomic mechanisms that control digestion. It includes many types of endocrine, paracrine, neurocrine, and autocrine regulators that control gastric emptying, motility, and the secretion of digestive enzymes. The endocrine signals also have the potential to control food intake. The main gut peptides that affect satiety are ghrelin, PYY (peptide YY), GLP-1 (glucagon-like peptide 1), and CCK (cholecystokinin) (Zanchi *et al.*, 2017). Ghrelin, which is mainly derived from the stomach, is the only known hunger-inducing gut peptide. PYY and GLP-1 are secreted by the enteroendocrine L cells, which are mainly located in the ileum and colon. CCK is secreted in response to dietary peptides and lipids, and it induces the secretion of pancreatic

hormones and bile. PYY, GLP-1 and CCK all increase satiety in their own ways, and acute administration of them decreases food intake in the short term (Dhillo, 2007).

Another important hormone that induces satiety is leptin. It is secreted by adipocytes and signals energy excess, having therefore an anorexigenic effect (Dhillo, 2007). Leptin directly effects the hypothalamus to influence food intake and endocrine function (Ronveaux *et al.*, 2015). Leptin also possibly regulates the neuronal responses to gastrointestinal hormones and interacts with them to induce short-term satiation (Ronveaux *et al.*, 2015). Leptin levels in the blood correlate with adipose tissue mass, so it signals the availability of energy stores and affects long-term energy intake (Dhillo, 2007). On the other hand, obese people possibly express leptin resistance, and leptin-deficient people and rodents are obese.

The digestive tract is in contact with the brain via the vagus nerve, which mediates bidirectional communication between the gut and the brain. The autonomous enteric nervous system regulates e.g. gut motility and gastric secretions, but communication with the brain is still essential. Vagal nerve endings are found in the lamina propria of the intestinal mucosa, and they can sense e.g. food volume (stretch) and gut hormones, and therefore signal the presence of nutrients to the brain (Waise *et al.*, 2018). Vagal signalling is transmitted to the nucleus tractus solitarius (NTS) of the brainstem, which is in contact with other brain regions, including the arcuate nucleus (Ahima & Antwi, 2008). The cell bodies of vagal nerves are located in nodose ganglia. Nodose ganglia have been found to express leptin receptors, so leptin can also affect vagal signalling (Ronveaux *et al.*, 2015). Vagal afferents in lamina propria express G-protein coupled receptors for e.g. CCK, GLP-1, PYY, and ghrelin, and activation of these receptors can cause neuronal innervation or inhibition (Waise *et al.*, 2018). It has been suggested that the innervation is calcium-dependent, and elevated calcium either causes action potentials or the signal is transmitted via a calcium wave (Waise *et al.*, 2018). Even though at least ghrelin and PYY causes changes in the activities of ARC neuropopulations, the satiety-inducing activities of the gut hormones GLP-1, PYY and ghrelin are possibly mainly vagally mediated, since their anorexigenic effects are abolished in vagotomy (Batterham *et al.*, 2002; Zanchi *et al.*, 2017; Waise *et al.*, 2018).

1.2.2. Short-chain fatty acids increase satiety

The effects of SCFA on food intake have been research a lot in rodents. Butyrate, propionate, and acetate can all reduce food intake, but their effects vary. Enteroendocrine L cells in the human colon

have been found to express SCFA receptors FFAR2 and FFAR3 (Karaki *et al.*, 2008; Tazoe *et al.*, 2009). SCFA can therefore stimulate GLP-1 and PYY release, but there is some difference between fatty acids. All SCFA increase GLP-1 secretion *in vitro* (Tolhurst *et al.*, 2012). However, in oral administration, acetate did not significantly increase GLP-1 and PYY, whereas butyrate increased the hormone levels the most (Lin *et al.*, 2012). The fact that butyrate and propionate but not acetate decrease gut hormone release could indicate that the effect is mediated by FFAR3. Still, many studies agree that the effect of SCFA on GLP-1 and PYY secretion is independent of FFAR3. FFAR3-deficient cells and animals do not express significantly decreased GLP-1 and PYY levels, and butyrate and propionate-dependent inhibition of food intake are present in FFAR3-deficient mice (Tolhurst *et al.*, 2012; Lin *et al.*, 2012; Psichas *et al.*, 2015). FFAR2-deficient animals, on the other hand, have lower baseline levels of GLP-1 and PYY (Tolhurst *et al.*, 2012; Lin *et al.*, 2012). Effects that are seen in FFAR3-deficient mice could be due to reduced FFAR2 expression (Tolhurst *et al.*, 2012).

SCFA have been associated with increased leptin production in adipocytes. *In vitro* studies show that butyrate, propionate, and acetate induce leptin production in adipocytes and that the effect could be FFAR3-dependent (Xiong *et al.*, 2004). Propionate elevated plasma leptin levels significantly in mice, but butyrate and acetate were not tested (Xiong *et al.*, 2004). The applicability of these results could be debated, since in some cases elevated SCFA is associated with decreased leptin levels (Gabriel & Fantuzzi, 2019). SCFA are not the primary stimulators of leptin production, since a high-fat diet stimulates weight gain and leptin production, and interventions related to SCFA modulate those effects (Gabriel & Fantuzzi, 2019). Therefore SCFA, at least propionate, could stimulate leptin production, but this might not be seen in all studies.

There is some evidence that SCFA could directly regulate the activity of the vagus nerve. FFAR3 has been found to be expressed in the nervous system in sympathetic ganglia (Kimura *et al.*, 2011) and both autonomic and sensory ganglia, including vagal ganglion (Nøhr *et al.*, 2015). FFAR3 is also expressed in the submucosal and myenteric ganglia of the enteric nerves, affecting the enteric nervous system (Nøhr *et al.*, 2013). The role of FFAR3-signalling in the nervous system still needs a lot more research, and only vague suggestions can be made. However, there is potential that SCFA have a broader direct effect on energy expenditure and homeostasis via the nervous system.

As mentioned before, the satiety-increasing effects of gut hormones are strongly mediated by the vagus nerve. This is also true for SCFA, since hepatic vagotomy and capsaicin treatment (which impairs capsaicin-sensitive sensory neurons) strongly attenuated the effects of intraperitoneally

administered acetate, propionate, and butyrate on food intake (Goswami *et al.*, 2018). Subdiaphragmatic vagotomy has also been shown to abolish the effects of butyrate on food intake (Li *et al.*, 2018). This indicates that even though SCFA could modulate the signalling of the orexigenic and anorexigenic ARC neurons of the hypothalamus, the effect is still somewhat dependent on the vagus nerve. Intraperitoneal injection of butyrate activated the nodose ganglion neurons (NGN) and the NTS *in vivo*, supporting the idea of a vagally mediated effect (Goswami *et al.*, 2018).

The activation of NGN and NTS by propionate and acetate were not studied, but as discussed earlier, acetate can directly reduce appetite via a central homeostatic mechanism. About 3 % of intravenously or colonically administered acetate ends up in the brain (Frost *et al.*, 2014). When acetate was administered into the third ventricle of the brain in rats, it reduced food intake 1-2 h after injection, but not as effectively as intraperitoneally administered acetate (Frost *et al.*, 2014). This indicates that even though acetate has a direct effect in the brain, it also has a systemic effect e.g. via the vagus nerve, which seems to be essential in the satiety-inducing effect of SCFA.

There are differences between the effects of SCFA on food intake. When mice were given a high-fat diet (HFD) supplemented with molarity-matched sodium salts of butyrate, propionate or acetate, butyrate suppressed food intake the most. Both butyrate and propionate inhibited HFD-associated weight gain, whereas acetate suppressed weight gain by 40 % (Lin *et al.*, 2012). This is in line with the fact that acetate did not stimulate GLP-1 and PYY release significantly whereas butyrate and propionate did (Lin *et al.*, 2012). The reduction in food intake by SCFA seems to occur in the rank order of butyrate > propionate > acetate in studies that include peroral or intraperitoneal (i.p.) administration methods and SCFA are given in equal molarities (Lin *et al.*, 2012; Goswami *et al.*, 2018).

When administered intravenously (i.v.), butyrate did not decrease food intake in mice on a HFD (Li *et al.*, 2018). Therefore, it could be speculated whether i.p. administration enables contact with colonic cells or vagal afferents and therefore enables a reduction in food intake while i.v. administration does not. The effects of i.v. propionate and acetate on food intake could be also interesting topics of research, since most research is focused on butyrate. It is also interesting how SCFA were able to suppress weight gain without significantly decreasing food intake, which is what acetate did in the study by Lin *et al.*, 2012. This could be due to their other metabolic effects, since e.g. butyrate improves adipose tissue composition and lipid and glucose metabolism, and these effects are only partly mediated by decreased food intake (Li *et al.*, 2018).

Even though there is clear evidence that SCFA can reduce food intake at least in rodents, the mechanisms are not completely understood. SCFA appear to increase satiety signalling, but satiety and the desire to eat do not always go hand in hand. Overeating can even change to loss-of-control eating in both rodents and humans especially if access to palatable food is most of the time restricted. This thesis studies the potential of SCFA to reduce loss-of-control eating. As a novel topic of research, this provides information about whether SCFA only decrease hunger or whether they also decrease energy intake even when eating is not driven by a physiological need.

1.3. Binge eating disorder (BED)

Binge eating disorder (BED) is an eating disorder characterized by binge eating episodes in which a person obsessively eats an unusually large amount of food in a relatively short period of time. Unlike in bulimia nervosa, BED patients do not use any compensatory mechanisms to decrease the weight gain following binge episodes, such as vomiting or using medication like laxatives. American Psychiatric Association has classified diagnostic criteria for BED in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, 2013), which are also used in the Finnish Current Care Guidelines (Eating disorders: Current Care Guidelines Abstract, 2014). Besides eating large amounts of food during a bingeing episode, a patient can eat too fast, eat until they feel uncomfortably full, eat a lot without being hungry, eat alone because of shame related to the amount of food, or feel self-loathing, depression or guilt after a bingeing episode. At least three of these additional criteria have to be met for diagnosis. Severe anxiety is also a typical part of BED and, in addition, bingeing episodes have to happen at least weekly for three months (Eating disorders: Current Care Guidelines Abstract, 2014). BED is also related to psychological and physical comorbidities such as depression, post-traumatic stress disorder and obesity-related disorders like type 2 diabetes and coronary heart disease (Eating disorders: Current Care Guidelines Abstract, 2014).

For people suffering from BED, treatment could include psychoeducation, psychotherapy, and only in some cases medication combined with psychotherapy. Medication usually helps only in the short term, so it is not prescribed routinely (Eating disorders: Current Care Guidelines Abstract, 2014). BED patients need to learn adequate, regular, and well-balanced eating and stop restricting food, since dieting and bingeing-related remorse and resentment only maintain the binge eating behaviour. Comorbidities related to BED, on the other hand, can and should be treated. Therefore,

this thesis study provides also a novel approach to treating BED in the future if SCFA are found to be safe and effective in attenuating bingeing episodes.

1.4. Modelling binge-like eating behaviour

Several rodent models have been established to model binge-like eating behaviour. All binge eating models have to include consumption of an unusually large amount of food in a relatively short period of time (Corwin *et al.*, 2011). The models include intermittent access to highly palatable foods including a lot of sugar or fat, or both, and controls consuming the same foods but without binge-like eating. Some rodent models also use food restriction to trigger food intake (Corwin & Babbs, 2012). This captures the fact that a person might restrict eating prior to bingeing, but on the other hand, bingeing can mean eating a large amount of food in the absence of hunger, which is what models without food restriction show. The length of the food restriction period can vary, and also the time of the day when palatable food is presented. Restriction during the dark time could increase consequent binge eating since rodents as nocturnal animals are most likely hungry at that time. Some models include a stress factor, such as a shock in the foot before access to palatable food (Corwin & Babbs, 2012). Food restriction might also provide an unpredictable variable that causes stress in the animals (Corwin & Babbs, 2012). Stress and anxiety increase binge-like eating behaviour and are also usually present in BED. As expected, every model has its benefits and shortcomings, and not everything can be combined in one model. Examples of different types of rodent models are listed in Table 1.

In this thesis study, a model of binge-like eating behaviour by Czyzyk *et al.*, 2010 was used. The model includes intermittent access to palatable, high-fat food (HFD) for 24 h once a week and does not include food restriction. In the model, mice binge eat during the day right after palatable food is presented, which is not a typical time for them to eat. Bingeing in the absence of hunger is a strength of this model. On the other hand, the mice do not experience stress, depression, or anxiety, which are characteristics of BED (Czyzyk *et al.*, 2010).

Table 1. Examples of binge-like eating models.

Model type	Authors	How it works	Advantages	Disadvantages
Intermittent access model, mice	Czyzyk *et al.*, 2010	Chow food *ad libitum*, in addition HFD 24 h/week	No food deprivation Bingeing not caused by hunger	Does not model stress/anxiety No weight gain
Intermittent access model, rats	Berner *et al.*, 2008	2 h/day sweet-fat chow followed by *ad libitum* standard chow	No food deprivation Weight gain occurs	Does not model stress/anxiety
History of restriction and stress, rats	Boggiano *et al.*, 2007	5 days of chow, amount 66% of control group + 2 days of Oreo cookies Shock in the foot before presenting the palatable food	Bingeing follows restrictive eating Includes stress, which increases bingeing	More harm to the animals Food deprivation No weight gain
Mild food restriction and the light-dark cycle, rats	Colantuoni *et al.*, 2002	Food deprivation for 12 h + palatable food 4 hours after lights off	Only mild restriction Increased intake of palatable food	Mild food deprivation No weight gain
Limited access, food restriction, and the light-dark cycle, rats	Bello *et al.*, 2009	22 h food deprivation + 2 h access to sweetened vegetable shortening, 2 h after lights off 2 non-consecutive days/week, other days chow *ad libitum*	Large energy intake after restriction	Food deprivation No weight gain

1.5. Different administration methods of SCFA

The administration methods of short-chain fatty acids can vary according to the aims of the research. When researching the effects of SCFA, the usual administration methods include i.p. injection, oral gavage, or peroral administration, in which SCFA are added to the food or drinking water. In some research colonic infusion, intravenous or intracerebroventricular methods have also been used (Frost *et al.*, 2014). The amounts of SCFA in the blood rise rapidly after i.p. injection since they are quickly absorbed into the circulation, but the concentrations also fall rapidly. SCFA are effectively metabolised in the liver and other tissues, so blood concentrations do not remain high. I.v. injection of 1.25 g/kg of sodium butyrate was determined to be the maximal tolerated dose in

mice, after which the blood concentration went back close to baseline in 45 minutes (Egorin *et al.*, 1999). I.p. injection of 1 g/kg sodium acetate increased plasma acetate levels significantly also for about 45 minutes (Shubitowski *et al.*, 2019). When sodium butyrate or sodium acetate were administered via oral gavage, their blood concentrations rose a significant amount, but at least 200 mM sodium acetate administration via drinking water did not affect plasma levels at all (Egorin *et al.*, 1999; Shubitowski *et al.*, 2019). Therefore, the more SCFA is given at once and the faster it can enter the circulation, the higher concentrations occur, but on the other hand the levels drop more rapidly.

The effects of SCFA on food intake are commonly studied with a SCFA-supplemented diet. Time periods during which body weight and food intake are followed are usually several weeks or even months long (Lin *et al.*, 2012; Li *et al.*, 2018). Administration via drinking water, on the other hand, is usually used when the effects on the gastrointestinal tract are studied. E.g., changes in T_{reg} cell activation can be studied via SCFA-supplemented drinking water (Smith *et al.*, 2013). In these cases, the drinking water is usually provided for a couple of weeks before the effects are evaluated. These types of studies are interested in long-term effects and changes in metabolism, which do not happen right away. I.p. and i.v. administrations and oral gavage, on the other hand, are used to see the acute effects (Kimura *et al.*, 2011; Frost *et al.*, 2014; Goswami *et al.*, 2018; Li *et al.*, 2018). The advantage of oral gavage is contact with the gastrointestinal tract, but i.p. injection helps reach higher concentrations at once.

2. Aims of the Study

Since SCFA have been found to decrease food intake in mice, the aim of this study was to evaluate whether butyrate, propionate or acetate also decrease energy intake in a mouse model of binge-like eating behaviour when administered intraperitoneally or via drinking water.

3. Materials and Methods

3.1. Animals

26 9-12 months old male (n=15) and female (n=11) C57BL/6N mice (Charles-River, Germany) were individually housed in individually ventilated cages with enrichments. Mice were acclimated for at least 1 week before initiating experiments and they were on a 12 h light-dark cycle with lights on

from 6 am to 6 pm, and the temperature was kept at 21-23 °C. Mice were habituated to handling and injections at least one week prior to the first SCFA experiment. Habituation lasted for three consecutive days and included lifting the mouse from its cage, putting it on top of another cage, immobilization, and two i.p. injections of 10 ml/kg 0.9% saline with 25G needles. Animal experiments were approved by the National Animal Experiment Board of Finland, according to EU directives harmonized with Finnish legislation. Animals were handled by professional caretakers and researchers with training in rodent experimentation. Welfare of the animals was taken into consideration when choosing the mouse model, and the model did not include restriction of food or water.

3.2. Experimental groups and induction of binge-like eating behaviour

The binge-like eating paradigm was used as developed by Czyzyk *et al.*, 2010 and described here as following: Mice were randomly divided into three groups (Chow, Continuous or Intermittent) with about the same gender distribution. Chow controls (n=8, five males and three females) had unlimited access to standard chow pellets (3.227 kcal/g, Altromin 1324, Lage, Germany). Continuous controls (n=9, five males and four females) had *ad libitum* access to both the standard chow pellets and high-fat diet (HFD) pellets (4.496 kcal/g, Altromin C1090-45, Lage, Germany), in which 45% of the energy is derived from fat. The Intermittent group (n=9, five males and four females) had *ad libitum* access to standard chow and intermittent access to HFD for 24 h per week, which is when they expressed binge-like eating. The first binge cycle was initiated as shown in Figure 4 by providing the mice with a 48-hour free choice of both chow and HFD, after which the mice received only chow for five days. HFD was then provided for 24 hours as a free choice between HFD and chow. For subsequent binge cycles, mice received only chow for 5-8 days followed by free choice between chow and HFD for 24 h. HFD was provided 4-8 hours after lights were turned on. Food pellets were provided on a metal feeding rack with a divider to separate chow and HFD, and the location of the chow food was randomized. The mice were weighed weekly.

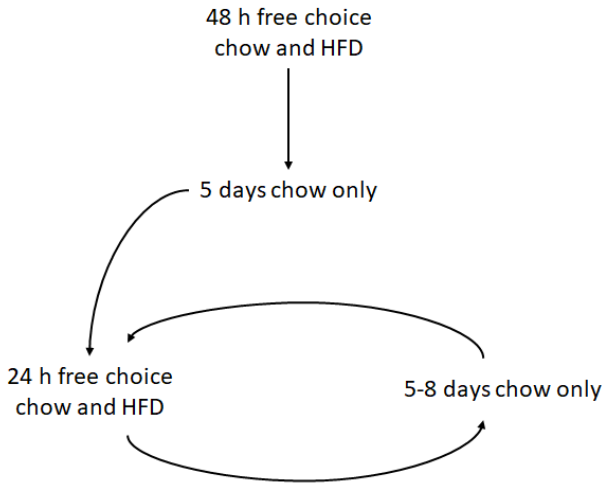


Figure 4. Inducing binge-like eating in mice.

3.3. Testing the model

Before initiating any experiments, the model was tested without administration of SCFA. The Intermittent group received HFD before noon, after which chow and HFD intakes were followed by scale at 1 h, 2.5 h and 24 h timepoints in all experimental groups. Energy intakes were calculated based on the changes in food weight. Energy intake was also measured at the same time on days 2, 3, 4 and 7 in order to see how daily energy intakes change and whether there are differences between groups.

3.4. Administration of SCFA via i.p. injection

Sodium butyrate was purchased from Acros Organics (Geel, Belgium), and sodium acetate and sodium propionate from Alfa Aesar (Kandel, Germany). The effects of different SCFA on binge-like eating were tested once per week according to the binge-like eating cycles. On each week of the experimentation, half of the mice in each experimental group received 1 g/kg body weight of sodium butyrate, sodium propionate or sodium acetate as a 10 ml/kg intraperitoneal injection, and the other half received a 10 ml/kg 0.9% saline injection as a vehicle. Dosages were determined according to other research, and the aim was to give a high but well-tolerated dose (Egorin *et al.*, 1999; Kimura *et al.*, 2011; Shubitowski *et al.*, 2019). Fatty acid solutions were prepared on the same day with injections. Fatty acids were dissolved in 0.9% saline, pH was checked with Fisherbrand pH-Fix 0-14 strips and adjusted to about 7 with 1 N HCl. Injections were given simultaneously with HFD, and food intake was measured by scale 1 h, 2.5 h and 24 h after injections, as described in Figure 5. Energy intakes were calculated based on food intakes. Food intake was also measured a day after

the HFD was removed to see how the mice would compensate for the excess energy intake. Experiment with butyrate was done as a cross-over study so that the mice that received butyrate one week received vehicle another week, and vice versa. Experiments with propionate and acetate were not repeated.

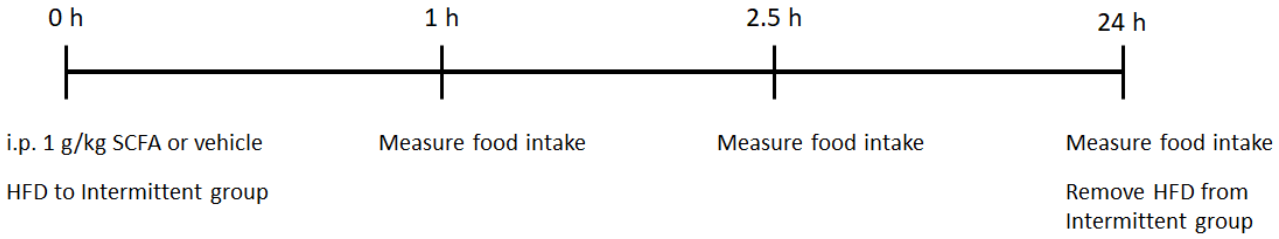


Figure 5. Time course of the i.p. SCFA experiments.

3.5. Administration of SCFA via drinking water

Sodium butyrate, sodium propionate and sodium acetate were dissolved in tap water as 200 mM solutions the same day they were given to the mice. Dosages were determined according to other research (Smith *et al.*, 2013; Shubitowski *et al.*, 2019). pH was measured with a Metrohm 744 pH meter and adjusted to 7 with 36% HCl. The vehicle solution was sodium-matched tap water with 200 mM NaCl. Mice received the drinking water (DW) with butyrate, propionate or acetate or vehicle 2 days before the binge-like eating experiment in order for them to get used to drinking it and so that the fatty acids would act in the body already before the bingeing would start. The experimental solutions were provided as the only choice of water in 10 ml tubes, and water intake was measured by volume at the same time with food intake. DW intake was measured in all except one DW butyrate experiment. Leakage was measured in five empty cages but not taken into account in the results. Drinking water was changed back to regular tap water after the binge eating experiment was finished, so the mice drank the experimental solution for three days in total. Food intake was measured daily after the mice received the experimental solution until one day after the experiment, so for four days in total. Food intake was also measured by scale 1 h and 2.5 h after the start of the bingeing experiment. The study design is described in Figure 6. Energy intakes were calculated based on food intakes. Drinking water experiments were all done as cross-over studies.

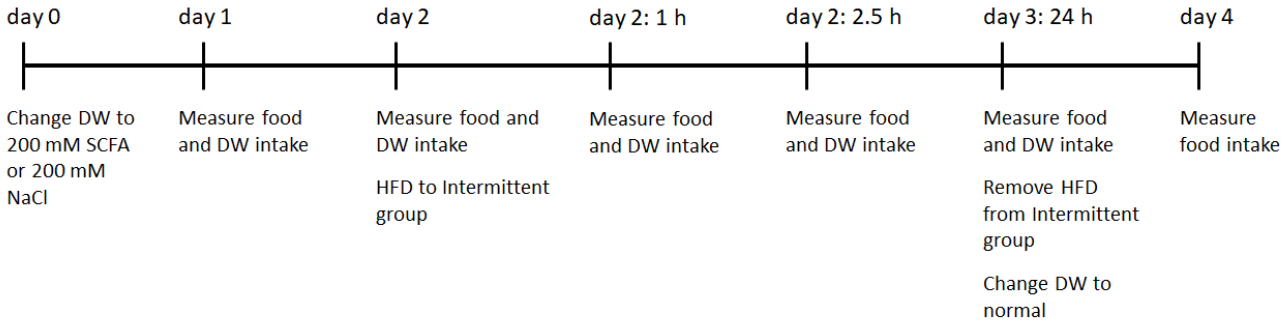


Figure 6. Time course of the DW SCFA experiments.

3.6. Adjusting the measured food weights

Food intakes were measured by scale throughout the experimentation, but the weight of both chow and HFD pellets changed during the experiments most likely due to changing humidity. Therefore, five empty cages with both chow and HFD pellets were added in the same rack with the animal cages, and the pellets were weighed daily when food intakes were measured. The average percentual change in food weight in the empty cages was calculated and used to derive a coefficient, by which the food weight was multiplied the second day. This was done each day separately to chow and HFD food in order to rule out changes in food weight. If a food intake measurement gave a negative value after this adjustment, it was due to slight scale inaccuracy and it was set to 0.

3.7. Statistical analyses

The energy intakes in SCFA groups and vehicle groups were compared with Student's t-tests in Microsoft Excel for Office 365 MSO. In studies with a parallel design (i.p. propionate, i.p. acetate, and when testing the model), unpaired 2-sample t-tests were used, whereas in cross-over studies (i.p. butyrate, DW butyrate, DW propionate and DW acetate) paired 2-sided t-tests were used. T-tests were done to compare energy intakes in mice that received SCFA and mice that received vehicle in the same timepoints (1 h, 2.5 h and 24 h) within an experimental group. In DW experiments, also daily energy intakes were compared with paired t-tests in all groups on days 1-4. When testing the model, all experimental groups were compared to each other in all timepoints. The results were visualised with GraphPad Prism 9.

Table 2. Sample sizes and reasons for exclusion in each experiment.

Experiment	Study design	Experimental group	Intended n	Timepoint	Final n	Reason for exclusion
Testing the model		Chow	8	all	8	
		Continuous	9	1 h, 2.5 h, day 1-2	9	
				day 3-4	8	1 mouse euthanized due to an abdominal tumor
		Intermittent	9	all	9	
i.p. butyrate	Cross-over	Chow	8	all	8	
		Continuous	8	1 h, 2.5 h	8	
				24 h	7	1 mouse excluded as an outlier in food intake
		Intermittent	9	all	9	
i.p. propionate	Parallel	Chow	4	all	4	
		Continuous	4	all	4	
		Intermittent propionate	5	all	5	
		Intermittent vehicle	4	all	4	
i.p. acetate	Parallel	Chow	4	all	4	
		Continuous	4	all	4	
		Intermittent acetate	4	all	4	
		Intermittent vehicle	5	all	5	
DW butyrate	Cross-over	Chow	8	day 1	7	1 mouse euthanized before cross-over
				day 2-4	6	1 mouse euthanized before cross-over 1 mouse excluded because the drinking bottle got displaced
		Continuous	8	day 1-2	8	
				day 3-4	7	1 mouse excluded as an outlier in food intake
		Intermittent	9	all	9	
DW propionate	Cross-over	Chow	7	all	7	
		Continuous	8	day 1	7	1 mouse excluded due to a mistake in measuring food intake

				day 3	7	1 mouse excluded as an outlier in food intake
				day 2 and day 4	8	
		Intermittent	9	all	9	
DW acetate	Cross-over	Chow	7	all	7	
		Continuous	8	day 1	8	
				day 2-4	7	1 mouse excluded as an outlier in food intake
		Intermittent	9	day 1-3	9	
				day 4	7	2 mice excluded because HFD was mistakenly not removed after 24 h

Some mice were excluded from some experiments. The sample sizes for each experiment and reasons for exclusion are listed in Table 2. Also, one male mouse in the Continuous group was euthanized while testing the model due to an abdominal tumor. One female mouse from the Chow group was euthanized during drinking water experiments due to unhealed skin problems.

The effects of different fatty acids on energy intake were compared with ANOVA or equivalent tests using IBM SPSS Statistics 26 and GraphPad Prism 9. For these statistical analyses, values representing how much a fatty acid decreased energy intake were calculated for the mice in each experiment. In other words, results from SCFA groups were normalized by using results from the corresponding vehicle groups. For the i.p. SCFA experiments, the value was the difference between energy intake after SCFA injection and the average energy intake of the whole vehicle Intermittent group, and it was calculated for 1 h, 2.5 h and 24 h timepoints for every mouse that received SCFA in the Intermittent group. For the DW SCFA experiments, since they all had a cross-over design, values for each mouse were calculated by the difference between energy intake when drinking SCFA and energy intake when drinking vehicle. These values were calculated for the Intermittent group in 1 h, 2.5 h and 24 h measurements, and also for Chow and Continuous groups in day 3 measurements. With the calculated values, the effects of different SCFA on energy intake during a bingeing episode were compared, separately in each timepoint. In the DW experiments, the effects of each SCFA were also compared in different groups (Chow vs. Continuous vs. Intermittent) on day 3. Tests of normality were done using Kolmogorov-Smirnov and Shapiro-Wilk tests in Prism, and Levene's test for homogeneity of variances in was done in SPSS. When the values were normally

distributed and variances were homogenous, ANOVA was used in SPSS. When the variances were non-homogenous, both Welch and Brown-Forsythe robust tests for equality of means were used in SPSS.

Statistical tests were also made to see whether changes in energy intake would correlate with water intake in the DW experiments in order to find out dose-dependence. The correlation between the change in energy intake by SCFA (energy intake after SCFA minus energy intake after vehicle) and DW intake was tested in Prism. Tests of normality using Kolmogorov-Smirnov and Shapiro-Wilk tests were done prior to correlation tests in Prism. If the values for both water intake and change in energy intake were normally distributed, Pearson correlation was used. If water intake or change in energy intake did not pass the test of normality, Spearman correlation was used. Correlation was tested in each experimental group on day 1, day 2, and day 3 for butyrate, propionate, and acetate in Prism.

4. Results

This research studied the effects of short-chain fatty acids on binge-like eating behaviour in a mouse model. The effects of butyrate, propionate and acetate on binge-like eating were tested with administration via 1 g/kg intraperitoneal injection (i.p.) or 200 mM drinking water (DW).

4.1. The experimental groups showed clear characteristics in feeding behaviour

The model was tested before administering any fatty acids in order to see whether it works the way Czyzyk *et al.*, 2010 described. As seen in Figure 7, the mice consumed primarily HFD whenever it was provided. The Intermittent group ate a lot during the first 1 and 2.5 hours (Figure 7A and 7B) even though they were not hungry. There was a statistical difference ($p<0.05$) between all the experimental groups in 1 h and 2.5 h measurements. By the 24 h measurement, the Intermittent group had eaten even twice as much as the control groups (Figure 7C). Daily energy intakes were compared throughout the week, and there was a statistical difference in energy intake between Continuous and Chow groups on all days except day 1 (Figure 7D). The Intermittent group consumed a lot of energy during day 1, which was the bingeing day, and ate less on days 2 and 3. The energy intake went back to the level of the Chow group on day 4 (Figure 7D). Energy consumption should therefore be on the usual level by the time of the next experiment. As seen in Figure 7E, weekly energy intake was higher in the Continuous group compared to Chow and Intermittent groups,

which had a similar level of energy intake. The Continuous group consumed mostly HFD. And even though the Intermittent group consumed HFD for only one day per week, HFD covered even 31 % of the weekly energy intake, which shows that the mice ate a lot when the HFD was available and afterwards compensated by eating less chow food.

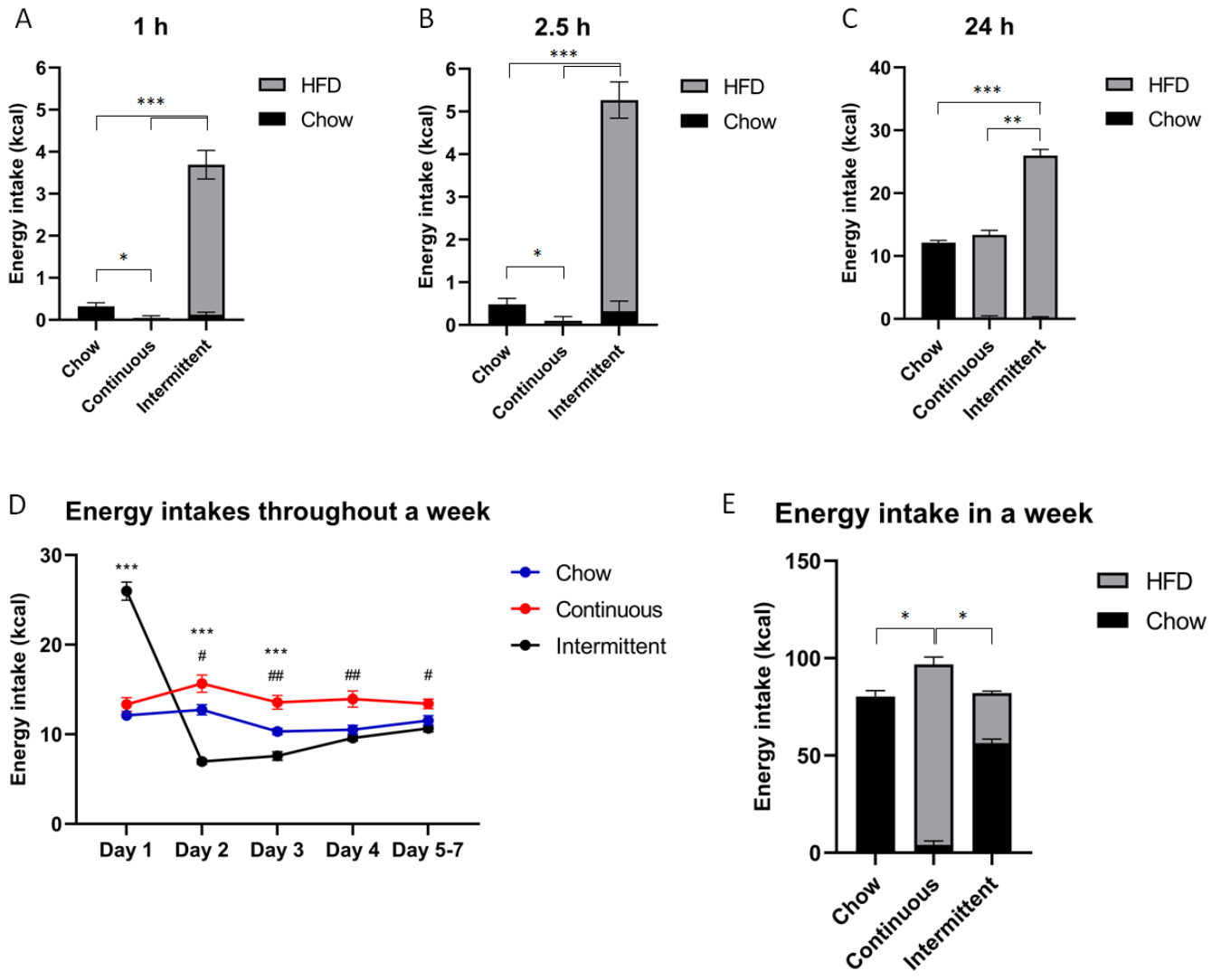


Figure 7. Testing the model. The Intermittent group started eating right away and consumed a lot during the bingeing day but compensated afterwards by eating less. Hence, the Continuous group had the largest overall energy intake in a week. Error bars: s.e.m.

A-B: Energy intakes 1 h (A) and 2.5 h (B) after giving HFD to the Intermittent group. *p<0.05, ***p<0.001

C: Energy intake during 24 h when the Intermittent groups received HFD. **p<0.01, ***p<0.001.

D: Energy intake throughout a week in each group. ***p<0.001 when comparing the Intermittent group to the other groups; # p<0.05 and ## p<0.01 when comparing the Continuous group to the other groups.

E: Overall energy intake in a week. *p<0.05.

HFD: high-fat diet.

4.2. Intraperitoneal SCFA decreased short-term energy intake markedly and 24 h energy intake slightly

I.p. administration of 1 g/kg butyrate, propionate and acetate decreased energy intake in the Intermittent group in 1 h timepoint (p=0.0017 for butyrate, p=0.023 for propionate, and p=0.011 for acetate) (Figure 8). In 1 h timepoint, butyrate decreased energy intake by 68% (Figure 8A), propionate by 57% (Figure 8D), and acetate by 62% (Figure 8G), so there was not much difference between the fatty acids. In 2.5 h timepoint, butyrate decreased energy intake by 33% (Figure 8B), propionate by 30% (Figure 8E) and acetate by 42% (Figure 8H). These results were however nonsignificant (p=0.064, p=0.061, and p=0.078, respectively). Between the three SCFA in 1 h and 2.5 h timepoints, there was no difference in decreasing energy intake (Table 3). None of the SCFA changed energy intake in Chow and Continuous groups in 1 h and 2.5 h timepoints.

Propionate and butyrate had also some effects on 24 h energy intakes when administered intraperitoneally (Figure 8C and 8F). Propionate reduced energy intake in the Intermittent group by 15% (p=0.042) and butyrate by 8.1 % (p=0.027) during the 24 hours after administration of the fatty acids. Acetate did not decrease energy intake after 24 hours (Figure 8I), and none of the fatty acids had statistically significant effects on energy intake in Chow and Continuous groups (p>0.05). When comparing the effects of butyrate, propionate, and acetate on 24 h energy intake with ANOVA, no statistically significant results were found (Table 3).

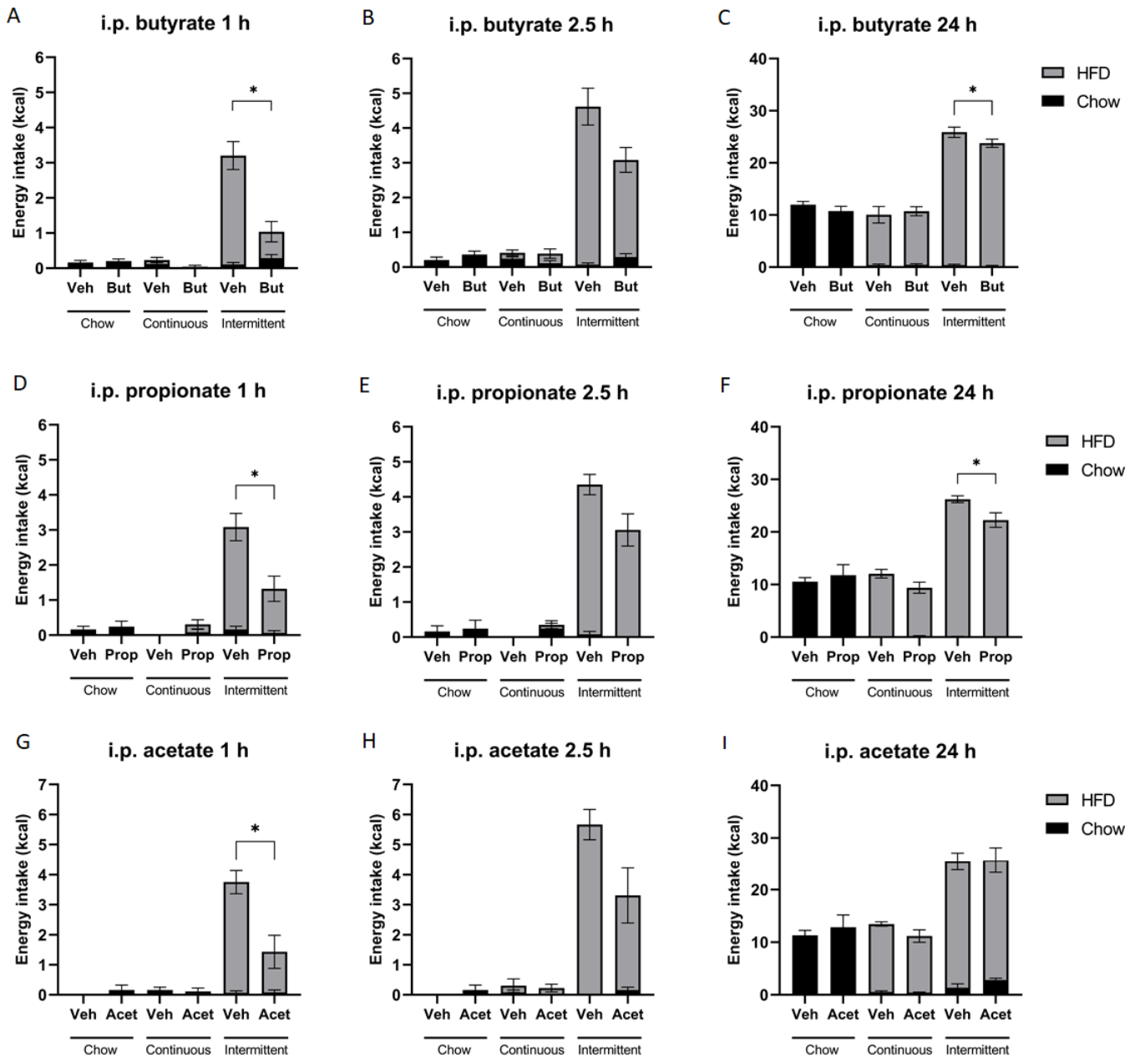


Figure 8. SCFA reduced energy intake during the first 1 h of the bingeing episode in the Intermittent group after intraperitoneal administration, and butyrate and propionate also reduced 24 h energy intake in the same group. Mice received 1 g/kg sodium butyrate (A-C), sodium propionate (D-F) and sodium acetate (G-I) or vehicle (0.9% saline) via 10 ml/kg i.p. injections. The Intermittent group received HFD at the same time with injections. Error bars: s.e.m. *p<0.05.

A-B: Energy intake in the Intermittent group 1 h (A) and 2.5 h (B) after i.p. injection of butyrate or vehicle.

C: Energy intake in all groups 24 h after i.p. injection of butyrate or vehicle.

D-E: Energy intake in the Intermittent group 1 h (D) and 2.5 h (E) after i.p. injection of propionate or vehicle.

F: Energy intake in all groups 24 h after i.p. injection of propionate or vehicle.

G-H: Energy intake in the Intermittent group 1 h (G) and 2.5 h (H) after i.p. injection of acetate or vehicle.

I: Energy intake in all groups 24 h after i.p. injection of acetate or vehicle.

HFD: high-fat diet. Veh: vehicle. But: butyrate. Prop: propionate. Acet: acetate.

4.3. SCFA in drinking water reduced 24 h energy intake in some groups

Butyrate, propionate, and acetate were administered via drinking water for three days altogether and energy intake was measured daily. Results are shown in Figure 9. Looking at Figures 9A-B, 9G-H, and 9M-N, it seems that the fatty acids slightly decreased energy intake at 1 h and 2.5 h timepoints in the Intermittent group, but none of the differences were statistically significant ($p>0.1$ in all groups).

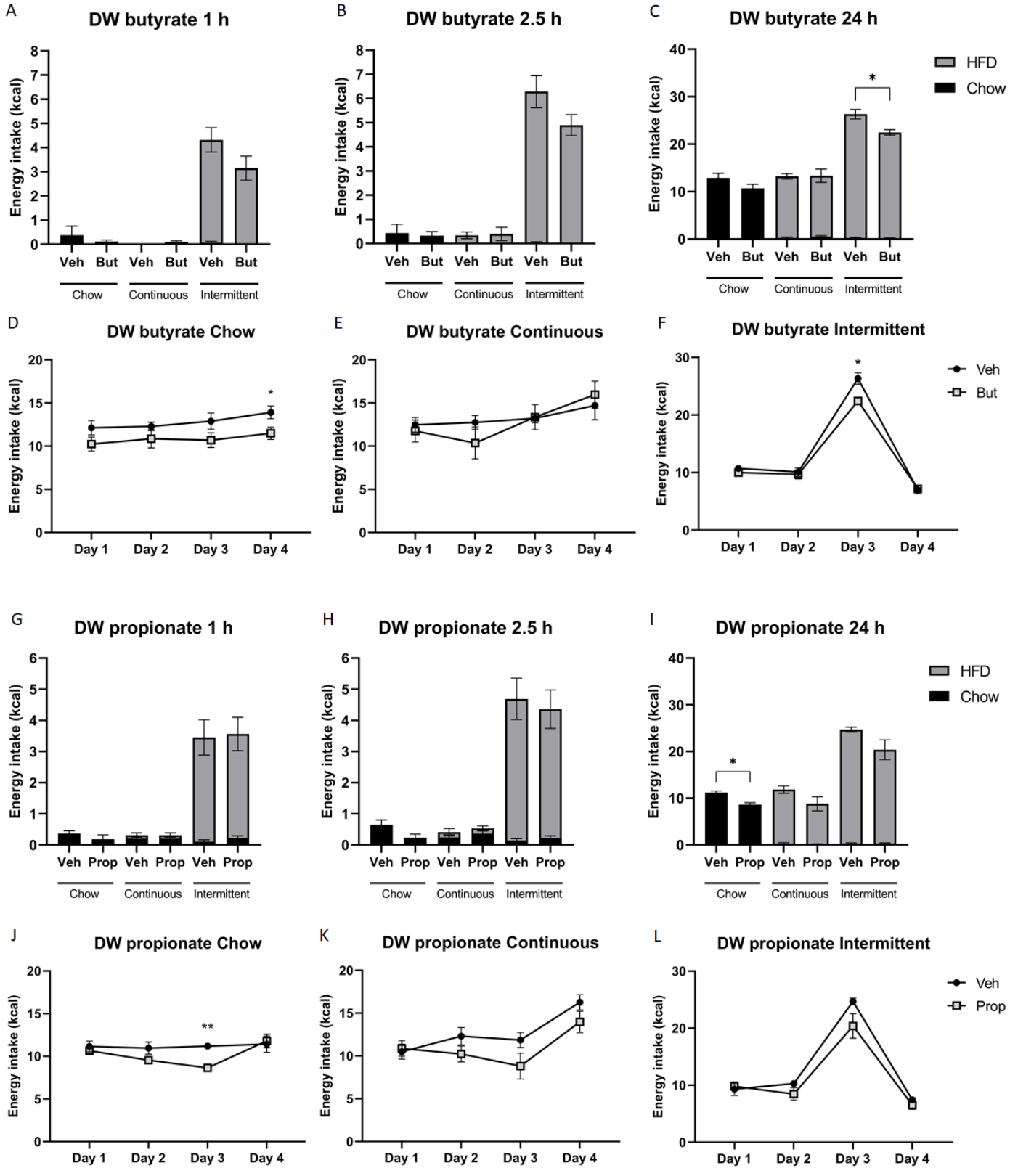
In Figure 9, 24 h measurements for each fatty acid are the same as day 3 energy intakes. Butyrate had a statistically significant effect on energy intake in the Intermittent group in the 24 h measurement ($p=0.017$) (Figure 9C). In the Chow group, butyrate had some effect on energy intake on day 3 ($p=0.054$) and day 4 ($p=0.045$) (Figure 9D). When mice in the Chow (Figure 9D) and Continuous (Figure 9E) groups drank water with 200 mM butyrate, some days they had lower energy intakes compared to when they drank vehicle solution. However, statistical tests did not give other significant results.

The same effect was seen with propionate in Chow, Continuous and Intermittent groups (Figure 9J-L), but propionate only decreased energy intake a statistically significant amount in the Chow group on day 3 ($p=0.0085$). Some differences in energy intakes were close to statistical significance: Continuous day 2 ($p=0.060$) and day 3 ($p=0.059$), and Intermittent day 3 ($p=0.089$).

When drinking 200 mM acetate solution, energy intake was lower throughout the experiment in the Continuous (Figure 9Q) and Intermittent (Figure 9R) groups compared to vehicle. It seemed that acetate did not have any effect on the Chow group (Figure 9P). In the Continuous group, acetate decreased energy intake on day 3 a statistically significant amount ($p=0.013$), which is seen in both Figure 9O and 9Q. Acetate had some effects on energy intake in some groups, but these were not statistically significant: Continuous day 2 ($p=0.075$), and Intermittent day 2 ($p=0.076$) and day 3 ($p=0.073$).

DW intakes were also measured by volume, and on one of the butyrate experiments the mice drank butyrate on average 2.56 ml (SD 1.58) and vehicle 5.61 ml (SD 1.70) per day. In propionate experiments the mice drank propionate on average 3.20 ml (SD 1.90) and vehicle 4.30 ml (SD 1.90) per day. In acetate experiments the mice drank acetate on average 4.50 ml (SD 2.50) and vehicle 5.50 ml (SD 2.50 ml) per day. This would mean that the average dosages of SCFA would be 56 mg/day for butyrate, 61 mg/day for propionate and 74 mg/day for acetate. Leakage was not considered,

and in the butyrate experiment the DW intake was measured only on the second half of the experiment.



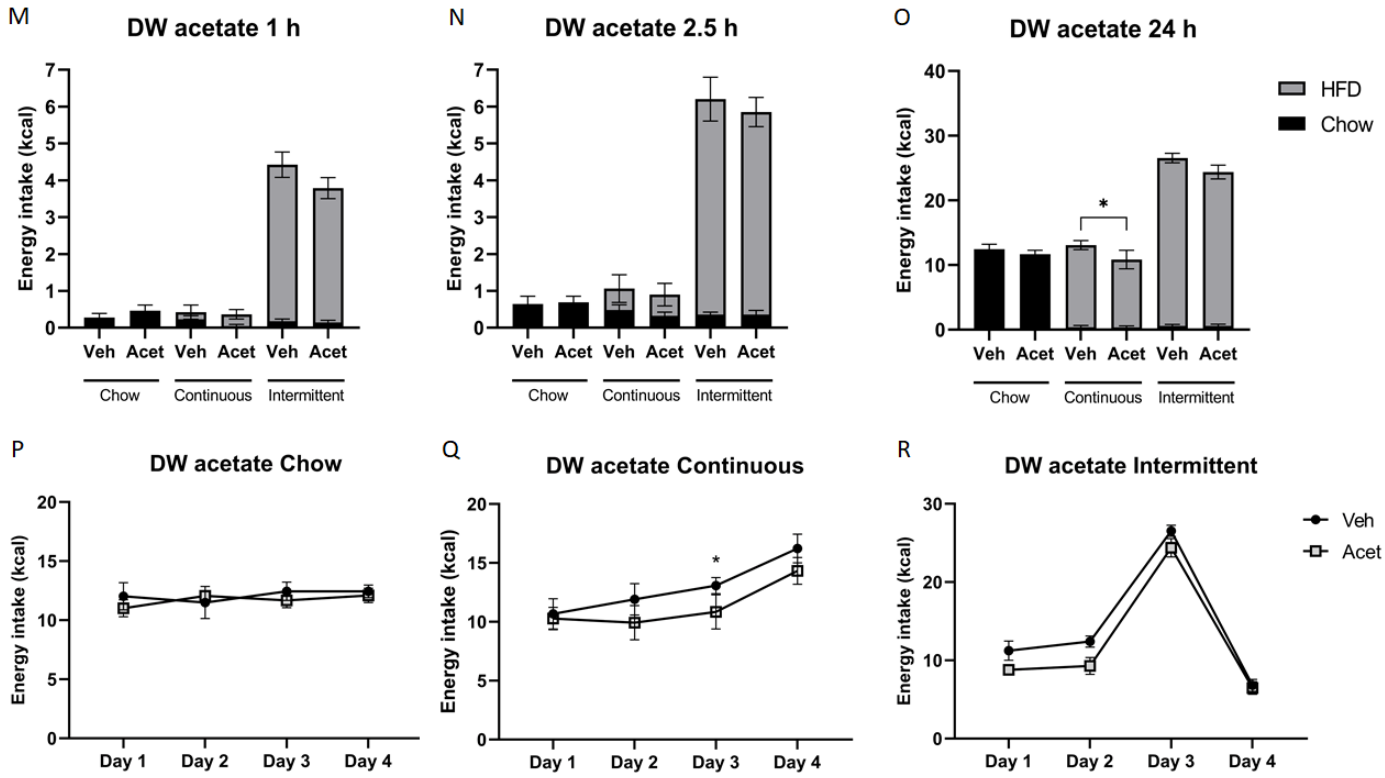


Figure 9. SCFA did not reduce short-term energy intake during administration via drinking water but decreased daily energy intake in some groups. Mice received 200 mM sodium butyrate (A-F), sodium propionate (G-L) or sodium acetate (M-R) and vehicle (200 mM NaCl) via drinking water. SCFA or vehicle drinking water was given to all groups on days 1-3, and the Intermittent group received HFD on day 3. Error bars: s.e.m. *p<0.05, **p<0.01.

A-C: Energy intakes 1 h (A), 2.5 h (B) and 24 h (C) after giving HFD to the Intermittent group while drinking butyrate or vehicle solution.

D-F: Energy intake in the Chow (D), Continuous (E) and Intermittent (F) groups while drinking butyrate or vehicle solution.

G-I: Energy intakes 1 h (G), 2.5 h (H) and 24 h (I) after giving HFD to the Intermittent group while drinking propionate or vehicle solution.

J-L: Energy intake in the Chow (J), Continuous (K) and Intermittent (L) groups while drinking propionate or vehicle solution.

M-O: Energy intakes 1 h (M), 2.5 h (N) and 24 h (O) after giving HFD to the Intermittent group while drinking acetate or vehicle solution.

P-R: Energy intake in the Chow (P), Continuous (Q) and Intermittent (R) groups while drinking acetate or vehicle solution.

DW: drinking water. HFD: high-fat diet. Veh: vehicle. But: butyrate. Prop: propionate. Acet: acetate.

4.4. Differences between the SCFA or their effects in experimental groups were not found

Statistical tests were performed in order to see whether the effects of butyrate, propionate and acetate differ from each other. Either ANOVA or Welch and Brown-Forsythe tests were used, and the results are shown in Table 3. For i.p. SCFA experiments, the effects of the three fatty acids on

energy intake were compared in the Intermittent group. Tests were done separately for 1 h, 2.5 h and 24 h measurements. The results showed that there was no difference between fatty acids at any timepoint, even though butyrate and propionate had a statistically significant effect on energy intake in the 24 h measurement and acetate did not.

Table 3. Comparing the effects of butyrate, propionate and acetate on energy intake and the effects of fatty acids in different groups.

Experiment	**Group**	**Timepoint**	**Data normally distributed? (Kolmogorov-Smirnov and Shapiro-Wilk tests)**	**Equal variances? (Levene's test)**	**Statistical test(s)**	**p value**
i.p. butyrate vs. propionate vs. acetate	Intermittent	1 h	no	yes	Welch	0.63
					Brown-Forsythe	0.62
		2.5 h	yes	yes	ANOVA	0.45
		24 h	yes	yes	ANOVA	0.14
DW butyrate vs. propionate vs. acetate	Intermittent	1 h	yes	yes	ANOVA	0.46
		2.5 h	yes	yes	ANOVA	0.62
		24 h/day 3	yes	yes	ANOVA	0.61
	Continuous	day 3	yes	yes	ANOVA	0.21
	Chow	day 3	yes	yes	ANOVA	0.33
DW butyrate Chow vs. Continuous vs. Intermittent		day 3	yes	yes	ANOVA	0.091
DW propionate Chow vs. Continuous vs. Intermittent		day 3	yes	yes	ANOVA	0.79
DW acetate Chow vs. Continuous vs. Intermittent		day 3	yes	yes	ANOVA	0.59

The same statistical analyses were made for DW SCFA experiments (Table 3). When comparing the effects of different fatty acids on energy intakes in 1 h, 2.5 h and 24 h timepoints in the Intermittent group, the results were nonsignificant. The effects of butyrate, propionate and acetate were also compared in the Chow and Continuous groups on day 3, and the results were nonsignificant. The effects of fatty acids on energy intake were also compared between Chow, Continuous and Intermittent groups on day 3 with ANOVA (for each SCFA separately), but the results were nonsignificant, indicating that the responses were not group dependent.

4.5. Correlation between DW intake and decrease in energy intake was not found

Correlation tests were done in order to see whether the decrease in energy intake was dose dependent in DW experiments, so whether DW intake correlates with energy intake. The results are shown in Table 4. Correlation was only found in butyrate experiments in the Chow group on day 3 ($p=0.043$) and Intermittent group on day 3 ($p=0.0090$). No other correlations were found. Also, if there had been dose-dependence, the correlation coefficient (r) should have been negative because of the way the change in energy intake was calculated, and this was not the case. The fact that DW intake was only measured in the second part of the cross-over in the butyrate experiment led to a small sample size in the correlation tests, so the results could be affected by chance.

Table 4. In most experiments there was no correlation between the decrease in food intake and DW intake.

Experiment	Experimental group	Timepoint	Data normally distributed?	n	Pearson/Spearman correlation	p value (r)
DW butyrate	Chow	day 1	yes	4	Pearson	0.065 (0.93)
		day 2	yes	3	Pearson	>0.1
		day 3	yes	4	Pearson	**0.043** (1.0)
	Continuous	day 1	no	4	Spearman	>0.1
		day 2	yes	4	Pearson	>0.1
		day 3	yes	4	Pearson	>0.1
	Intermittent	day 1	yes	4	Pearson	>0.1
		day 2	yes	4	Pearson	>0.1
		day 3	yes	4	Pearson	**0.0090** (0.99)
DW propionate	Chow	day 1	no	7	Spearman	>0.1
		day 2	no	6	Spearman	>0.1
		day 3	yes	6	Pearson	>0.1
	Continuous	day 1	no	8	Spearman	>0.1
		day 2	yes	8	Pearson	>0.1
		day 3	yes	7	Pearson	0.072 (0.71)
	Intermittent	day 1	no	9	Spearman	>0.1
		day 2	yes	9	Pearson	>0.1
		day 3	yes	9	Pearson	>0.1
DW acetate	Chow	day 1	yes	7	Pearson	>0.1
		day 2	no	7	Spearman	>0.1
		day 3	yes	7	Pearson	>0.1
	Continuous	day 1	no	7	Spearman	>0.1
		day 2	yes	7	Pearson	0.067 (0.72)
		day 3	yes	7	Pearson	>0.1
	Intermittent	day 1	no	9	Spearman	0.067 (0.65)
		day 2	no	9	Spearman	>0.1
		day 3	yes	9	Pearson	>0.1

4.6. Body weight increased only in the Continuous group

The mice were weighed weekly, and the progression of body weight in each experimental group is shown in Figure 10. The weights of the mice in Chow and Intermittent groups stayed similar throughout the experiments, but the Continuous group gained weight. This was expected based on the average weekly energy intakes of the groups when the model was tested, since energy intake was higher in the Continuous group compared to Chow and Intermittent groups (Figure 7). The weight difference between Chow and Continuous groups was statistically significant on weeks 3-10 and between Continuous and Intermittent groups on weeks 3-12. Towards the end, the average

weight of the Continuous group decreased and there was no longer statistical difference between groups.

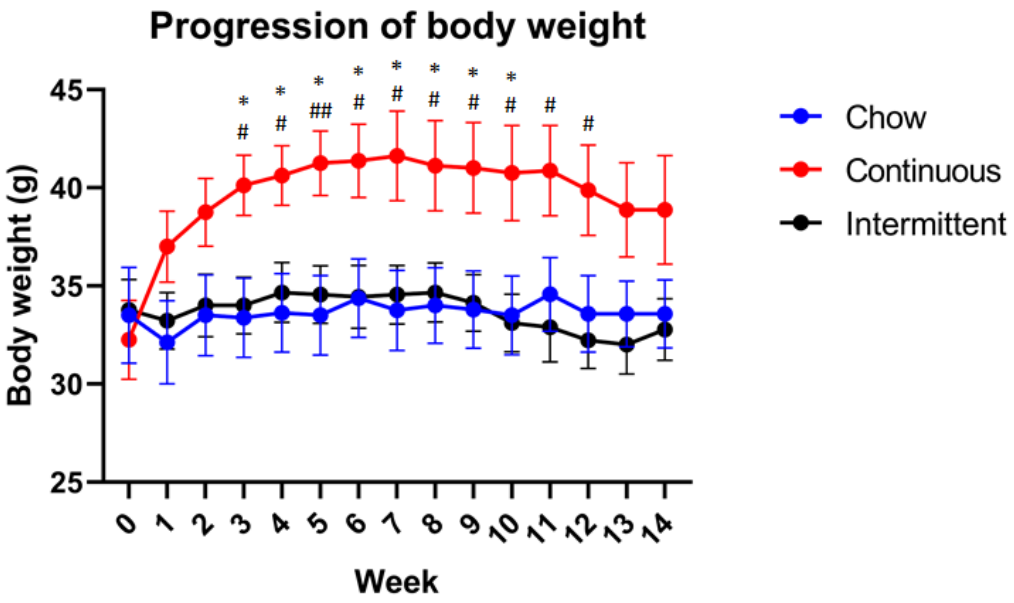


Figure 10. Average body weights of the mice in the Chow (blue), Continuous (red), and Intermittent (black) groups throughout the experimentation. *p<0.05 between Continuous and Chow group. #p<0.05 and ##p<0.01 between Continuous and Intermittent group. Error bars: s.e.m.

5. Discussion

This study shows that short-chain fatty acids have a substantial effect on binge-like eating behaviour in the short term, but long-term effects vary. SCFA had stronger and more consistent effects after i.p. administration compared to drinking water, which is likely caused by the high transient concentrations. Butyrate, propionate, and acetate all had similar effects on energy intake after i.p. administration at 1 h and 2.5 h timepoints, but butyrate had the most substantial effect at 1 h and acetate at 2.5 h timepoints. An interesting difference between the SCFA was seen at the 24 h timepoint, in which acetate did not decrease energy intake but butyrate and propionate did by 8.1% and 15%, respectively. Therefore, butyrate and propionate affected the whole bingeing episode and had a longer-lasting effect than acetate, even though they had likely all been metabolized after 24 hours. The fact that butyrate and propionate had a more substantial effect on energy intake than acetate is in line with Goswami *et al.*, 2018, even though the rank order of butyrate > propionate > acetate in reducing overall energy intake was not seen in this study. Still, none of the differing effects were confirmed by the statistical analyses. The rather small sample size was enough to show the

strong effects of SCFA on energy intake but likely not large enough to compare the effects between different fatty acids.

SCFA are sources of energy, but their energy content does not explain decreases in food intake. The average weight of the mice in the Intermittent group was about 33.7 g, which means that they received on average 33.7 mg of SCFA intraperitoneally. This would translate to about 0.20 kcal for butyrate, 0.17 kcal for propionate, and 0.12 kcal for acetate. These amounts were only 3.1%-6.2% of the energy intake at 1 h, whereas the SCFA decreased energy intake a lot more. In DW experiments, the daily dosages were a little larger but the energy contributions of SCFA were still minimal compared to the whole day energy intake.

In the drinking water experiments, SCFA decreased energy intake or did not change it, but most results were nonsignificant. The variation in the results between mice was considerable and most of them did not correlate with DW intake unlike expected. Therefore, the effects between mice were not explained by different dosages. If the experiments were to be repeated with different mice, the results may change. It seemed that when SCFA decreased food intake in some experimental groups, the effect was strongest on day 3 (24 h measurement), which is when the mice had consumed the SCFA solution the longest and most likely the greatest amount. Drinking consistently increased towards day 3 and also towards the end of the whole experimentation, which means that the mice got more used to drinking DW with SCFA (data not shown). SCFA could have been therefore administered for a more extended time period for more clear results, but that would have increased excess sodium intake, which could have been harmful to the mice in the long run.

The leakage of water tubes could have been a problem when testing for dose-dependence. Leakage was followed with the empty cages and it seemed to vary a lot between tubes. Since there was a lot of variation in leakages and it could not be measured in individual water tubes, it was not considered in the results. Not knowing the leakage could make studying dose-dependence more difficult. The fact that no dose-dependence was not found could challenge the physiological relevance of the results of the DW experiments.

Intraperitoneal SCFA decreased energy intake only in the Intermittent group, whereas in DW experiments, some fatty acids decreased energy intake in Chow and Continuous groups, as well. In i.p. experiments, Chow and Continuous groups did not eat at the time of administration, but in the DW experiments they received SCFA throughout the day. This indicates that SCFA have to be present

when most of the feeding occurs. Also, the fact that there was decrease in energy intake in the Chow and Continuous groups as well means that the SCFA worked as expected, i.e. they decreased energy intake by increasing satiety. Administration via drinking water may not increase blood concentrations significantly, but the mechanisms of increasing satiety may be independent of blood concentrations, which has at least been found for butyrate by Li *et al*. On the other hand, giving SCFA perorally for a more extended time period could have beneficial effects on metabolism and decrease energy intake in all experimental groups, but based on these results, it cannot be said whether it would help decrease binge eating.

There were differences between groups in how the drinking water affected energy intakes. In all of the DW experiments, the day 1 energy intake of the Continuous vehicle group was on the same level as day 1 energy intake of the Chow vehicle group. When the experiment continued, the energy intake of the Continuous group increased, which mostly did not happen in the Chow group as was seen in Figure 9. This effect was especially seen in the propionate and acetate experiments. The effect could be explained by the sodium-rich, not very tasty drinking water that leaves the mice thirsty, which could attenuate the habit of overeating until the mice get used to drinking it.

In this study, the mouse model by (Czyzyk *et al.*, 2010) worked as expected. Binge eating cycles remained relatively constant throughout the experimentation, which is a key strength of the model. The downsides of the model are that the binge-like eating group does not gain weight, which happens in binge eating disorder, because the mice compensate for excess energy intake. Using some other model of binge-like eating with more frequent access to palatable food, like that of Berner *et al.*, 2008, could solve this problem. Also, the mice do not experience stress or anxiety according to the authors, but the stress level was not studied right after HFD was removed, so the mice might still experience stress at some point. Differences in compensation were not seen between vehicle and SCFA, and no anticipation before receiving HFD was seen. The Continuous group does gain weight, but also their weight started decreasing towards the end. This could mean that the mice start eating less after a long time on the HFD, or that the SCFA or salty vehicle water that were served for three days per week decreased their food intake. The age of the mice could also play a role, since they were all at least one year old at the end.

If the study were to be repeated, younger mice could be chosen for the experiments, since in the original model by Czyzyk *et al.*, 2010 the mice were 10 weeks old. In the i.p. experiments no differences in energy intake were seen in the Chow and Continuous groups since they did not

normally eat during the day, so some mice in the Chow group could be moved to the Intermittent group by initiating binge-like eating. This would increase the sample size of the Intermittent group and increase the statistical significance of the results without using more animals. On the other hand, butyrate and propionate in drinking water had some effects on the energy intakes of the Chow and Continuous mice in some days, which means that taking mice away from the Chow group could lead to losing some interesting results in the DW experiments. Also, since mice eat so much HFD right away when they receive it, even an earlier timepoint for measuring food intake could be helpful.

These results indicate for the first time that SCFA may have the potential to reduce the metabolic effects of binge eating to some extent. If SCFA were administered as a high amount right before the bingeing behaviour would occur, they could decrease energy intake a lot. The effect is short term, but it could last for the bingeing episode. Chronic administration may not be beneficial since the dosage has to be high. Physiologically relevant amounts of SCFA or increasing their endogenous production may not be enough to affect the loss-of-control behaviour that is seen in BED patients, so giving prebiotics or fibre-rich foods that increase SCFA production to binge on may not work. If SCFA were to be given to humans, effects could vary according to different baseline productions of SCFA. Humans also have varied gut microbiomes and diets, unlike laboratory animals. SCFA could also be absorbed before they reach the gut where they affect gut hormone release, so injection of SCFA or giving them in a capsule that releases the SCFA in the gut could be considered. Also, giving BED patients short-chain fatty acids may not remove the issue, which is the psychological basis of the eating disorder. However, there could have positive psychological outcomes when the patient notices that the treatment works. The treatment could at least alleviate the risks caused by weight gain, such as the cardiovascular effects or type 2 diabetes.

The next step in research could be to test different dosages or administration methods, such as intragastric gavage or i.v. administration, or to look into what brain areas are affected by SCFA in binge-like eating behaviour. Future research could give more insight into the mechanisms of how SCFA affect binge-like eating behaviour, and whether the effect is via satiety regulation or if the SCFA affect overall loss-of-control or even addiction behaviour. E.g., the effects of SCFA on binge drinking could be tested.

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